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ORIGINAL ARTICLE

Genetic Bottlenecks of the Wild Chinese Giant Salamander in Karst Caves

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Abstract The rarity of limestone cave species due to habitat degradation makes them of special interest in conservation biology. The wild Chinese Giant Salamander *Andrias davidianus*, an evolutionarily distinct and globally endangered species, are nearly all obligate into living in inaccessible mountain caves now. We detected that only 14–29 breeders, with the effective population size of 9–25 (5–44 of 95% confidence interval), were in each of three caves, through genotyping 20 microsatellite loci on larvae that were flushed out of caves. Both heterzygosity excess and M (ratios of allele number to allele size range) tests indicated severe genetic bottlenecks among populations. Both mitochondrial, with only one or two haplotypes of D-loop region (770–771bp) in each population, and nuclear genetic structure showed clear divergence between populations. Considering the long life history, small population size, and genetic differentiationof the Chinese Giant Salamander, putting an end to poaching and recovering the karst ecosystem instead of releasing may be the sole measures to save this severely threated species.

Keywords limestone cave, small population, genetic drift, *Andrias davidianus*, subterranean habitat

1. Introduction

Subterranean environments are renowned as fragile and vulnerable ecosystems. Anthropogenic activities such as agriculture, urban development, waste disposal, deforestation, and resource extraction constitute environmental pressures leading to polluted aquifers, destroyed caves, denuded landscapes, and declining biodiversity (van Beynen *et al.*, 2012). Karst caves serve as shelters for a variety of plants and animals, some of which are endemic to caves and are referred to as troglobites (Culver and Pipan, 2009). Among vertebrates, few amphibians are restricted to karst caves, such as cave-dwelling salamanders *Proteus anguinus*, *Eurycea* spp., and *Necturus* sp. (Goricki *et al.*, 2012), but more typically, they utilize caves as one of a variety of suitable habitats for reproduction, refuge, and/or hibernation (Sket, 1997; Liu *et al.*, 2009; Niemiller and Miller, 2009; Köhler *et al.*, 2010; Liu, 2010). The rarity of cave species makes them of special interest in conservation biology.

The Chinese Giant Salamander (CGS) *Andrias davidianus* is the largest extant amphibian with a generation length of about 15 years. Historically, this species occurred widely in the watersheds of the Yellow, Yangtze, and Pearl River. However, it has experienced dramatic population declines in the past sixty years, estimated to be as much as 80% over the last three generations due to over-exploitation for the commercial luxury food trade (Liang *et al.*, 2004). To this end, it is now listed as critically endangered both in the Chinese Red Book of Amphibians and Reptiles and in the IUCN Red List of Threatened Species (Jiang *et al.*, 2016). Its current distribution is highly fragmented and several populations are already extirpated (Wang *et al.*, 2004). Nearly all wild individuals are dwelling in mountain caves where they breed (Luo *et al.*, 2009; Su *et al.*, 2009). Larvae produced inside the caves and flushed out in late winter and early spring might mature into adults in rivers;

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however, they have been heavily poached at the exit of caves with mist nets or electric fishing device in the past few years (Wang and Zhang, 2012). In addition, human-facilitated introductions but without pre-genetic screening also pose a genetic pollution threat (Murphy *et al.*, 2000).

For obligate cave dwellers, caves present formidable barriers to successful colonization and to movement between caves (Culver *et al.*, 2006). Most cave populations of CGS may also be isolated due to the barriers or threats to immigration, including dam construction, alteration of river courses, pollutions, and poaching, and are thus predicted to have risks of extinction under fluctuant circumstances due to genetic problems such as genetic drift and inbreeding depression (Frankham, 2005).

Genetic monitoring is costing less and being more sensitive and reliable than traditional monitoring approaches for conservation and management (Schwartz *et al.*, 2007). A recent reduction in abundance generates specific patterns of nucleotide variation over time compared with mutation drift equilibrium. Effective population size (*Ne*), that refers to the size of a theoretical, ideal population with the same genetic characteristics as the actual population, can be assessed on the basis of maximum likelihood estimations or Bayesian analysis (Wilson *et al.*, 2003; Kuhner, 2006). In addition, it is possible to detect recent bottlenecks based on detecting alleles' deviations from expectations under mutation drift equilibrium (Peery *et al.*, 2012; Hoban *et al.*, 2013).

Amphibian larvae in species where they are more accessible and abundant than adults are often sampled for population genetic study (e.g. Cosentino *et al.*, 2012; Peterman *et al.*, 2014). Although ideally CGS adults would have been sampled, low encounter rates of this rare species due to long-term poaching and lots of complicated shelters meant that this was impossible in karst habitats. Here, we identified full siblings of CGS larvae that flushed out of caves, addressed the effective population size and genetic structure of populations inside, and highlighted the urgency of conservation in subterranean habitats for this amphibian species most in need of conservation action, identified by the EDGE of Existence programme of the Zoological Society of London (Isaac *et al.*, 2012).

2. Materials and Methods

2.1 Study Sites and sampling The three sampled caves are illustrated in Figure 1. Only about 300-600 larvae were flushed out of the Shaliangzi cave (SLZ; Zhouzhi County, Shaanxi province), as described by Wang and Zhang (2012), in January-March of 2011 and 2012. The larvae that flushed out of the Taoyuan cave (TY; Nanjiang County, Sichuan province), with four exits at the same bank within a distance of 600 m, were around one thousand each year (local poachers, personal communication). The larvae that flushed out of the Yanxia cave (YX; Yanxia County, Guizhou province), located at the bank of a small stream, averaged 514 each year during 2004–2006 (Su *et al.*, 2009).

Tail tissue of 61 and 56 injured larvae that flushed out of the SLZ cave in 2011 (grouped as S11) and 2012 (grouped as S12), respectively, were provided by the local Fishery Bureau. Oral swabs were collected from 48 larvae collected by villagers from the TY cave during 2011–2012, and from 24 juveniles collected as larvae by farmers from the YX cave in the spring of 2008, following the procedure of Poschadel and Möller (2004). Tail tissue and oral swab samples were stored individually in 95% ethanol and frozen at –20°C prior to laboratory analysis.

2.2 DNA isolation and characterization Total genomic DNA was extracted using the *EasyPure*$^{\text{TM}}$ Genomic DNA kit (Beijing TransGen Biotech Co., Ltd.). The complete mitochondrial control region (*i.e.* D-loop, 770–771 bp) was amplified following the procedure of Tao *et al.* (2005). The PCR products were sequenced on ABI 3730 in both directions.

Variability of 20 microsatellites, including GS66, GS78, and GS132 locus (Meng *et al.*, 2008), CIBad01-CIBad16 loci (Wang *et al.*, 2014) and CIBad17 locus (with primers 5'-TTCTCACAGATGGAATCACA-3' and 5'-GAAGCAGCTATGCCGTAT-3'), were detected following the procedure of Wang *et al.* (2014).

2.3 Genetic diversity D-loop sequences were aligned in Mega v6.0 (Tamura *et al.*, 2013) and calculated the haplotype diversity (hd) and nucleotide diversity (π and θ) in DnaSP v5.10.1 (Librado and Rozas, 2009). In DnaSP v5.10.1, we also analyze D-loop haplotypes with the Tajima's *D* test, which is based on the comparison between the average pairwise difference and the number of polymorphic sites. If equilibrium is not reached after a demographic event, negative *D* values are expected under population expansion and positive *D* values are expected under population decline (Tajima, 1989).

Potential microsatellite genotyping errors were detected using Microchecker software v2.2.3 according to Brookfield's approach (Van Oosterhout *et al.*, 2004). We estimated allelic diversity, overall observed and expected heterozygosity, and tested for linkage disequilibrium between all possible locus pairs using Fisher's exact tests in GENEPOP 4.0 (Rousset, 2008). In GENEPOP, we also tested for deviations from Hardy-Weinberg frequencies, reflected as whether the inbreeding coefficient (*f*; Weir and Cockerham, 1984) is significantly different from zero; exact *P* values are calculated with the Markov chain method (dememorization 10 000, batches 100, iterations per batch 5000; Guo and Thomson, 1992). We performed a sequential Bonferroni correction to minimize type-I errors by adjusting *P*- values to correct for multiple table-wide tests (Rice, 1989).

We calculated the number of rare alleles (*Ar*) (defined as alleles with a frequency <1% in all 189 samples), and the frequencies of private alleles in each population. Allelic richness, which could be considered as an alternative criterion to measure genetic diversity (Rodrigáñez *et al.*, 2008), was standardized for variation in sample size and calculated based on the minimal sample size of 24 in this study.

2.4 Number of breeders and effective population size To provide an indication of population size inside the karst cave, sibships of larvae and *Ne* were reconstructed using the COLONY program (Jones and Wang, 2010), with sets of full-pedigree likelihood method, polygamous mating system of both sexes, no sibship size prior, allelic dropout rate of 0.01 and false allele rate of 0.01 for each locus. The same dataset was processed 8–16 runs with different random seeds to confirm the reliability of results.

2.5 Bottleneck tests Microsatellite data was analyzed with the heterozygosity excess test (Luikart *et al.*, 1998) and *M*-ratio test (Garza and Williamson, 2001). We used the Wilcoxon two-tailed test in BOTTLENECK (Piry *et al.*, 1999), as it is more powerful than the sign test when average sample size per site is 30 or less, and the standardized test requires a minimum of 20 loci (Luikar *et al.*, 1998). We chose a two-phase mutation model (TPM), which is modified from the strict stepwise mutation model (SMM), because it allows for (a user-defined percentage of) multi-step mutations and

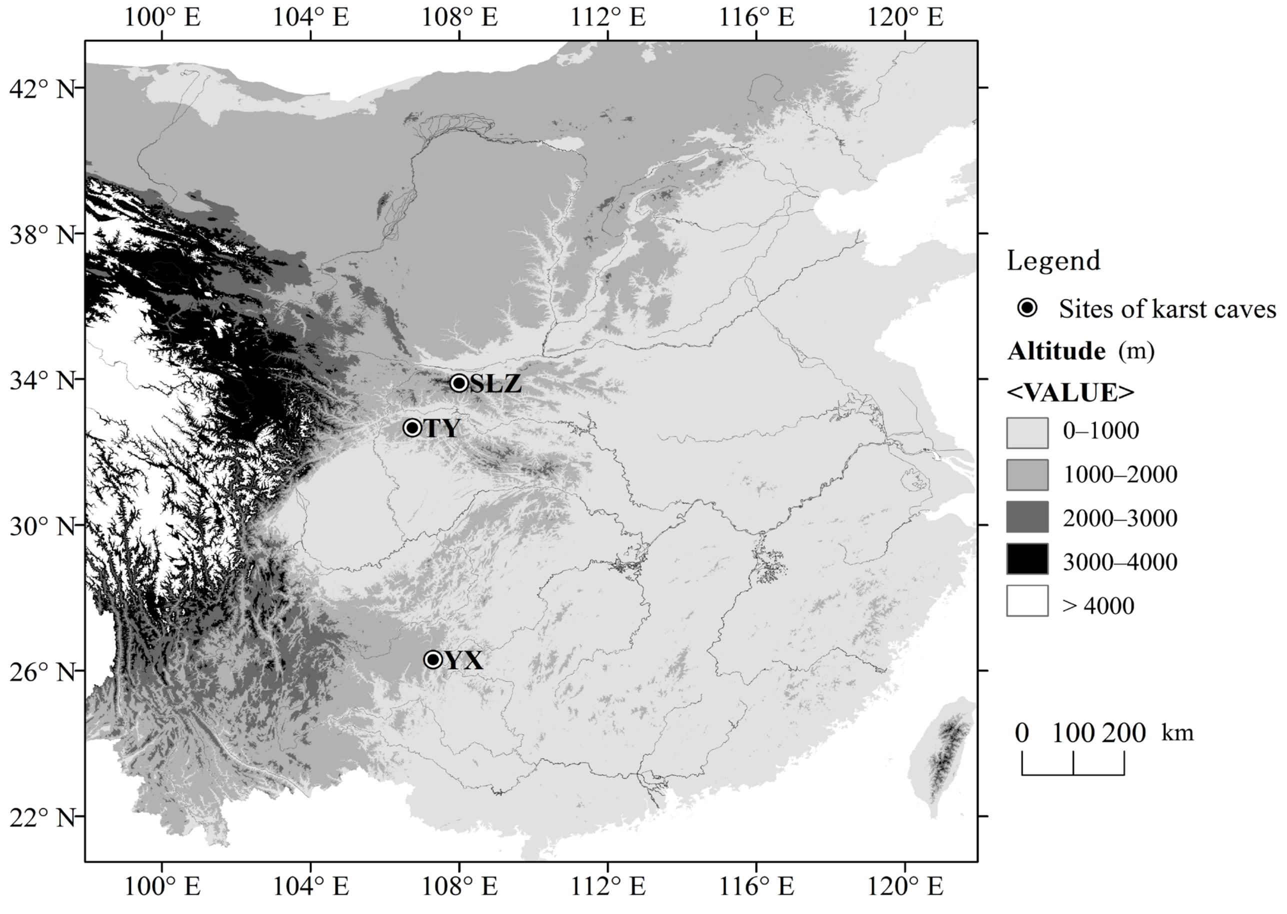

Figure 1 Position of the three sampled karst caves. SLZ: Shangliangzi, Zhouzhi County, Shaanxi Province; TY: Taoyuan, Nanjiang County, Sichuan Province; YX: Yanxia, Guizhou County, Guizhou Province.

consequently to be best fit for most microsatellite datasets (Piry *et al.*, 1999). In BOTTLENECK, we chose a 5% multi-step mutations (Piry *et al.*, 1999; Storfer *et al.*, 2014).

The *M*-ratio test is based on the frequency distribution of allelic sizes, which is expected to have gaps after a bottleneck due to stochastic loss of rare alleles. The *M*-ratio is computed in each data set as simply the ratio of the number of alleles per locus divided by allelic size range for that locus. Evidence of deviation from the null hypothesis of demographic stability can be concluded if the observed value is lower than a simple threshold value of 0.68 (Garza and Williamson, 2001), which is widely used as a "rule of thumb" in conservation genetics.

2.6 Population subdivision D-loop haplotypes in the three populations, together with other sequences of Cryptobranchidae species (GenBank code GQ368662, Zhang and Wake 2009; AB445787–99, AB445800–03, Matsui *et al.*, 2008) were used to reconstruct the gene genealogy using maximum likelihood (ML) as implemented in PhyML 3.0 (Guindon *et al.*, 2010). We use *Cryptobranchus alleganiensis* as an outgroup and conducted bootstrap analysis using 1000 ML bootstrap pseudoreplicates of the dataset, fixing model parameters at maximum likelihood values and estimating tree topology.

Clusters of individuals in the sampled populations were also inferred using microsatellite genotype data in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). Simulations were run using a burn-in period of 10 000 sweeps followed by 100 000 MCMC iterations. The number of possible clusters (K) was set from 1 to 12 (i.e. gene pools assumed), and 30 independent runs were repeated to guarantee convergence. Twenty independent runs with the highest log likelihood [Ln Pr(X/K)] of the posterior probability were selected to estimate the true K (Evanno *et al.*, 2005).

3. Results

3.1 Genetic diversity Two haplotypes with one transversion site were found in the D-loop region in the SLZ population, with the haplotype diversity (hd) and nucleotide diversity (π, θ_w) of 0.503, 0.00053 and 0.00022 in 2011, and 0.503, 0.00053 and 0.00023 in 2012, respectively. In contrast, one unique haplotype was found in the D-loop region in either TY or YX populations.

Micro-Checker revealed no evidence for null alleles of all microsatellite loci except GS132 and GS78 due to stuttering. The numbers of loci that deviated from Hardy-Weinberg equilibrium ($P < 0.05$) were 15 (S11), 16 (S12), 11 (TY), and 4 (YX), respectively (Table 1). Mean alleles in the populations were 6.8 (4–8, S11), 4.9 (2–7, S12), 4.9 (1–9, TY), and 3.0 (1–5, YX), respectively, with the corresponding allelic richness of 2.7 ± 0.1, 2.1 ± 0.1, 2.4 ± 0.2, and 3.0 ± 0.3, respectively.

3.2 Population size Based on the best sibship configuration in COLONY, N_e with 95% confidence interval was estimated to be 9 (5–24), 25 (15–44) and 17 (8–35) in the SLZ, TY, and YX caves, respectively (Table 2). All the 61 larvae that sampled in the SLZ cave during 2011 were assigned into 6 families (clusters) that come from 7 fathers and 7 mothers. Similarly, the 56 larvae that sampled in this cave during 2012 were assigned into only 2 families that come from 9 fathers and 8 mothers. Combined these two years, all the 117 larvae were estimated to come from 11 fathers and 10 mothers. The breeders in the TY and YX caves were estimated to be 29 and 15 breeders, respectively.

3.3 Bottleneck and genetic drift The Tajima's *D* values (*P*) of D-Loop haplotypes in SLZ were 2.35 and 2.28 in 2011 and 2012, respectively. In the SLZ population, all microsatellite loci except CIBad06 and CIBad08 reduced alleles from 2011 to 2012, with the allelic richness reduction of 22% (2.7→2.1). Only two loci (CIBad05-06) had one new allele in 2012. Significant heterozygosity excess ($P < 0.05$) was detected at 4, 5, 9, and 2 loci in S11, S12, TY, and YX, with the Wilcoxon *p* values of 0.648, 0.003, 0.000, and 0.196, respectively, indicating recent bottlenecks in these cave populations (Table 3). All *M* values at each locus in the three populations were lower than the threshold value (0.054–0.429 *vs.* 0.68, Table 3), also indicating serious population decline recently.

3.4 Genetic subdivision There were 16 variable nucleotide sites across the D-loop region for the three populations and the overall *p*-distance between populations is 0.011. The rooted ML phylogeny reveals that mtDNA haplotypes of CGS are separated into three reciprocally monophyletic clades (Figure 2). The TY population was closer to the SLZ population but further to the YX population (Figure 2). Compared with the D-loop haplotype in YX population, all haplotypes in SLZ and TY populations have one deletion A (*i.e.* 770 bp). STRUCTURE analysis revealed one peak at K=3 of ΔK value for the three *Andrias* populations (Figure 3a). When three clusters were assigned, all individuals have the admixture proportion of > 82% to be a cluster and no mixture between different populations (Figure 3b).

Table 1 Characterization of the 20 polymorphic microsatellite loci in the three cave populations of CGS.

Locus	*A*				A_p				A_r				Allelic richness				H_E				H_O				*P*			
	S11	S12	TY	YX	S11	S12	TY	YX	S11	S12	TY	YX	S11	S12	TY	YX	S11	S12	TY	YX	S11	S12	TY	YX	S11	S12	TY	YX
GS66	7	6	3	4	5	5	0	2	2	2	2	2	2.8	2.6	1.5	4.2	0.779	0.798	0.062	0.675	0.869	0.893	0.063	0.696	**0.047**	**0.001**	1.000	0.969
GS78	8	7	4	3	4	4	0	0	1	1	1	0	3.1	3.0	2.1	3.0	0.778	0.827	0.630	0.434	0.770	0.821	0.400	0.583	**0.000**	**0.000**	**0.000**	0.159
GS132	7	5	3	4	4	0	2	2	3	2	1	1	2.8	2.1	1.5	4.2	0.544	0.654	0.156	0.548	0.557	0.750	0.042	0.478	0.627	0.504	**0.000**	0.709
CIBad01	6	5	7	5	1	1	1	2	2	1	3	4	2.4	2.1	3.5	5.0	0.699	0.616	0.496	0.336	0.820	0.714	0.521	0.125	**0.000**	**0.000**	0.085	**0.000**
CIBad02	7	5	4	2	3	2	0	2	2	0	1	1	2.8	2.1	2.0	2.0	0.779	0.719	0.332	0.439	0.803	0.857	0.313	0.208	0.069	**0.018**	**0.028**	**0.015**
CIBad03	7	4	7	4	2	0	2	0	3	0	3	0	2.8	1.7	3.5	4.0	0.701	0.658	0.677	0.638	0.787	0.750	0.542	0.667	0.242	0.583	**0.011**	0.763
CIBad04	7	4	4	2	4	2	0	1	3	0	1	1	2.8	1.7	2.0	2.2	0.696	0.630	0.558	0.206	0.705	0.732	0.688	0.227	**0.002**	**0.009**	0.282	1.000
CIBad05	8	5	3	3	4	2	0	0	3	1	0	0	3.1	2.1	1.5	3.3	0.726	0.722	0.559	0.555	0.918	0.946	0.604	0.773	**0.021**	**0.001**	0.694	0.055
CIBad06	7	7	9	4	3	2	3	0	2	0	3	0	2.8	3.1	4.5	4.0	0.837	0.830	0.535	0.630	0.934	0.964	0.438	0.750	**0.005**	**0.000**	**0.000**	0.074
CIBad07	7	4	3	2	0	0	1	2	1	0	2	2	2.8	1.7	1.5	2.0	0.731	0.699	0.173	0.496	0.738	0.768	0.188	0.417	**0.001**	0.094	1.000	0.676
CIBad08	5	5	6	1	5	2	0	1	3	0	0	0	2.0	2.1	3.1	1.0	0.768	0.705	0.402	-	0.885	0.875	0.362	-	**0.001**	**0.000**	**0.013**	-
CIBad09	7	5	5	2	1	2	3	0	1	1	3	0	2.8	2.1	2.6	2.2	0.804	0.782	0.551	0.511	0.836	0.696	0.553	0.955	0.053	**0.000**	0.356	**0.000**
CIBad10	8	6	1	2	4	2	1	0	2	0	0	0	3.1	2.6	0.5	2.2	0.829	0.830	-	0.495	0.967	1.000	-	0.455	**0.000**	**0.000**	-	1.000
CIBad11	6	2	7	4	7	5	0	2	2	0	0	1	2.4	0.9	3.6	4.0	0.440	0.340	0.601	0.695	0.508	0.429	0.681	0.792	**0.024**	0.053	**0.018**	0.830
CIBad12	7	5	4	3	1	0	1	2	3	0	3	2	2.8	2.1	2.2	3.0	0.776	0.730	0.468	0.592	0.787	0.893	0.341	0.750	**0.000**	**0.000**	**0.011**	0.371
CIBad13	4	3	3	1	2	1	2	0	2	0	2	1	1.6	1.3	1.5	1.0	0.604	0.626	0.081	-	0.656	0.732	0.042	-	0.436	**0.002**	0.065	-
CIBad14	8	5	6	4	1	0	1	0	1	0	0	0	3.3	2.1	3.0	4.0	0.722	0.563	0.480	0.480	0.746	0.625	0.479	0.542	**0.000**	**0.012**	**0.001**	**0.021**
CIBad15	6	5	3	2	4	2	0	0	4	1	3	2	2.4	2.1	1.5	2.0	0.554	0.538	0.081	0.496	0.623	0.661	0.083	0.667	**0.001**	0.055	1.000	0.113
CIBad16	6	4	6	5	5	4	2	2	3	2	1	0	2.4	1.7	3.0	5.0	0.609	0.518	0.745	0.530	0.705	0.679	0.667	0.667	**0.000**	**0.024**	**0.007**	0.789
CIBad17	8	6	9	2	3	3	1	2	2	1	2	3	3.1	2.6	4.5	2.0	0.820	0.757	0.696	0.403	0.820	0.786	0.604	0.458	**0.000**	**0.012**	**0.018**	0.637
Average	6.8	4.9	4.9	3.0	3.15	1.95	1.00	1.00	2.25	0.60	1.55	1.00	2.7	2.1	2.5	3.0	0.710	0.677	0.436	0.509	0.772	0.779	0.401	0.567				

The number of alleles (*A*), private alleles (A_p) and rare alleles (A_r, with the frequency of < 5% in all 189 samples), allelic richness (standardized in sample size of 24), expected (H_e) and observed (H_o) heterozygosities, and *P*-value of Hardy-Weinberg exact test were based on the sample from SLZ (S11, S12: $n = 61$ and 56 respectively in 2011 and 2012), TY ($n = 48$), and YX ($n = 24$).

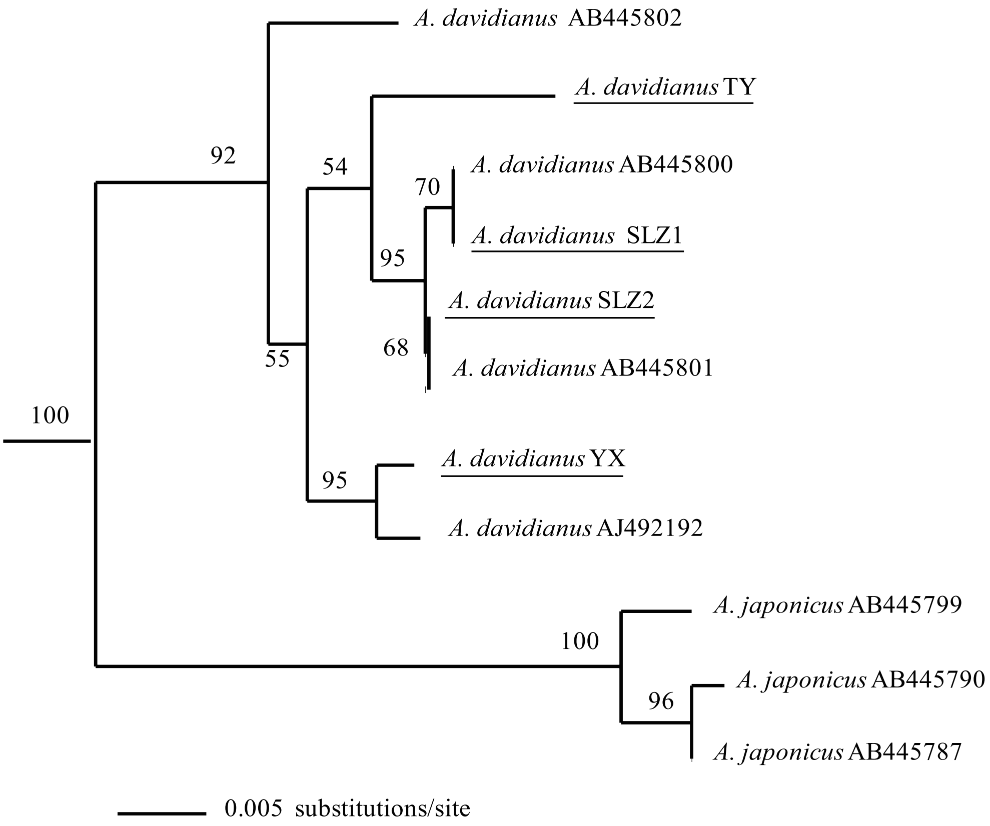

Figure 2 Midpoint-rooted maximum likelihood (ML) phylogeny of Chinese giant salamander mtDNA D-loop haplotypes. The numbers on branches represent ML bootstrap values (1000 replicates). *Cryptobranchus alleganiensis* are used as an outgroup.

4. Discussions

4.1 Genetic variance and population subdivision Only one or two D-loop haplotypes were detected in the three sampled populations. Similarly, only one to three haplotypes of Cytb or D-loop were found in each population of Japanese giant salamander (Matsui *et al.*, 2008) and North American hellbender (Sabatino and Routman, 2009). However, at least 27 haplotypes of D-loop were detected in CGS until now (Tao *et al.* 2005), indicating that the mitochondrial genetic variation of Chinese giant salamander mainly happens between geographic units but not within populations.

However, a much higher haplotype diversity (hd, 0.842–1.000 *vs.* 0.503) and nucleotide diversity (0.00496–0.01114 *vs.* 0.00065) were found in previous studies (Tao *et al.* 2005; Fang *et al.* 2008; Table 4), as their samples were from a much wider region (such as the upper Yangtze River; Tao *et al.*, 2005) or from farms, where adults from far different rivers were stocked together.

Previous studies showed genetic divergence of CGS in China even though no distinct geographic structure were found partially due to human relocation (Murphy *et al.*, 2000; Tao *et al.*, 2005; Tao *et al.*, 2006; Yang *et al.*, 2011). Only one D-Loop haplotype was found in the TY and YX population, and two haplotypes differentiated with only one nucleotide site were found in the SLZ population, which may indicate that no introduced genome existed in our cave populations at present. However, the clear genetic divergence among the three cave populations in terms of mitochondrial (Figure 2) and nuclear polymorphism (Figure 3) further support the downstream associated detrimental genetic pollution due to governmental-oriented releasing activates.

4.2 Small population size and genetic drift Based on some sporadically published breeding parameters, including brood amount of 400 eggs/kg for mother, egg fertility rate of 70%, and embryonic hatchability of 5%, Su *et al.* (2009) estimated that the sum weight of female breeders were 37 kg inside the YX cave. Considering that the female breeders (0.35–3.5 kg; Wang *et al.*, 2000) may reach large size (*e.g.* 1.5 kg) in caves because of the absence of poaching, the estimation of Su *et al.* (2009) were three times to our sibship analysis (24 *vs.* 7 mothers, Table 2). One reason may be that their hatchability was underestimated (e.g. > 10% of *A. japonicus*, Suzuki *et al.*, 2000).

Populations with a large *Ne* are resistant to loss of

Table 2 Sibship clusters and effective population size (*Ne*) analysis using COLONY program based on the microsatellite genotypes of larvae from SLZ, TY and YX.

	SLZ			TY	YX
	2011	2012	2011+2012		
Sample size	61	56	117	48	24
No. of fathers	7	9	11	17	8
No. of mothers	7	8	10	12	7
Total parents	14	17	21	29	15
Clusters	6	2	6	1	1
Ne (95% CI)	9 (5–24)	8 (4–23)	9 (5–24)	25 (15–44)	17 (8~35)

Table 3 Results from BOTTLENECK (TPM model) and estimates of *M* (the ratio of number of alleles per locus divided by allelic size rage for that locus), as an estimate of putative bottlenecks for populations of SLZ (S11, S12: *n* = 61 and 56 respectively in 2011 and 2012), TY (*n* = 48), and YX (*n* = 24).

Locus	Probability under TPM				*M*			
	S11	S12	TY	YX	S11	S12	TY	YX
GS66	0.339	**0.046**	**0.013**	0.263	0.156	0.133	0.333	0.066
GS78	0.419	**0.043**	0.365	0.330	0.276	0.280	0.308	0.333
GS132	**0.016**	0.454	0.062	0.264	0.226	0.161	0.429	0.308
CIBad01	0.410	0.319	**0.004**	**0.006**	0.207	0.172	0.280	0.122
CIBad02	0.332	0.233	0.056	0.236	0.212	0.152	0.235	0.154
CIBad03	0.201	0.220	0.123	0.425	0.241	0.235	0.171	0.190
CIBad04	0.204	0.337	0.389	0.462	0.156	0.235	0.308	0.069
CIBad05	0.162	0.212	0.213	0.320	0.242	0.135	0.333	0.103
CIBad06	**0.012**	**0.034**	**0.001**	0.463	0.212	0.189	0.220	0.190
CIBad07	0.354	0.072	0.068	0.103	0.171	0.190	0.143	0.154
CIBad08	**0.028**	0.298	**0.005**		0.294	0.200	0.162	--
CIBad09	0.149	**0.013**	0.137	**0.038**	0.171	0.152	0.200	0.222
CIBad10	0.145	**0.001**		0.116	0.178	0.133	--	0.118
CIBad11	**0.013**	0.301	**0.033**	0.162	0.240	0.222	0.171	0.098
CIBad12	0.360	0.174	0.171	0.191	0.212	0.172	0.138	0.176
CIBad13	0.427	0.074	**0.020**		0.190	0.231	0.333	--
CIBad14	0.143	0.166	**0.021**	0.145	0.163	0.102	0.240	0.190
CIBad15	0.057	0.128	**0.022**	0.103	0.113	0.152	0.333	0.222
CIBad16	0.124	0.279	0.359	0.068	0.182	0.190	0.146	0.111
CIBad17	0.221	0.269	**0.028**	0.292	0.242	0.207	0.170	0.054
Wilcoxon (*P*)	0.648	**0.003**	**0.000**	0.196				

genetic diversity through genetic drift and inbreeding. It is estimated that the minimum viable *Ne* should not be less than 50 in the short term and should be greater than 500 in the long term to maintain genetic diversity (Jamieson and Allendorf, 2012). If we use this as a guide, it is likely that the sampled cave populations (with *Ne* < 44) will decline in genetic diversity rapidly. In the SLZ population, we found that *Ne* reduced from 9 to 8 and the allelic richness declined at a faster rate than heterozygosity (2.7→2.1 *vs.* 0.710→0.671), primarily due to the loss of rare alleles (2.25→0.60; Table 1). More alleles (1→5) deviated from the mutation-drift equilibrium also showed that this population decreased to a threshold and face genetic bottlenecks. Arguably it is

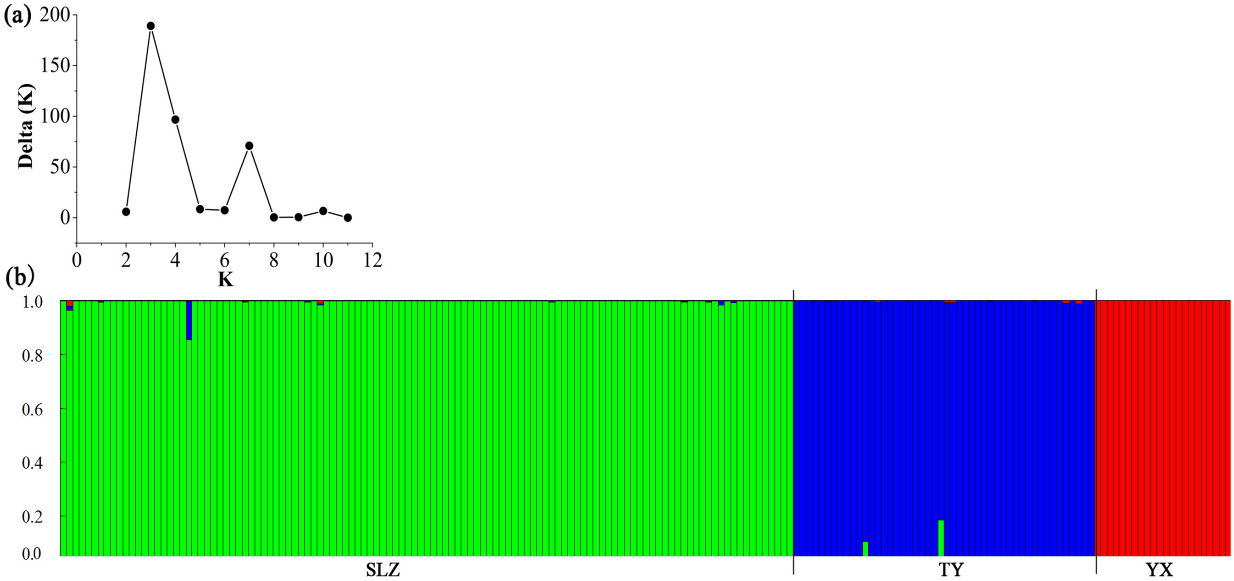

Figure 3 Clustering assignment depending on the Bayesian method under an admixture model obtained by STRUCTURE software. (a) ΔK values as a function of K based on 20 runs, indicating the most likely number of three genetic clusters, (b) Output of genetic clusters identified, each individual is represented by a single column that is divided into segments whose size and color correspond to the relative proportion of the animal genome corresponding to a particular cluster. Populations are separated by black lines..

allelic richness rather than heterozygosity that reflects the long-term evolutionary potential of a population, since it is the number of genetic variants in a population that determines the material available for natural selection to act upon (Tracy *et al.*, 2011; Weiser *et al.*, 2013). Recent meta-analyses have shown that the loss of diversity at neutral loci following a population bottleneck is often accompanied by an equivalent or greater loss of diversity at other highly variable adaptive genetic loci, such as major histocompatibility complex (MHC) genes that are important in disease resistance (Radwan *et al.*, 2010; Sutton *et al.*, 2011). Field surveys indicated that the larvae production of wild populations decreased sharply throughout its range and even disappeared at some sites in recent years (Luo *et al.*, 2009; Su *et al.*, 2009; Wang and Zhang, 2012). If this is the case, our results suggest managers to make concrete efforts to maintain allelic richness and enlarge effective population size for the long-term viability of cave populations.

4.3 Conservation suggestions Some reservoirs or rivers were reported to find large adults in recent years, but such places, polluted with domestic sewage or agricultural chemicals or out of this species' range (such as Beijing and Shandong Province), were usually unfit for breeding and seemed to harbor released or escaped captive-bred individuals. The natural breeding caves like SYZ, TY, and YX provide very important refuges to CGS. The larvae that flushed out caves are important recruitment to cave populations and need to keep away from poaching for farming stock.

Subterranean ecosystems generally exhibit little or no primary productivity and rely almost entirely on resources produced outside caves (Culver and Pipan, 2009). No larvae were flushed out of the YX cave after the water inlet was blocked with concretes to prevent poisoning in 2008, which obviously changed the inner hydrological conditions and cut off the food resources of breeders inside. Floods are important sources of organic input into cave systems, however, it may reduce ovipositon sites or cause asphyxiation of embryos. These could be the possible hypotheses why larvae were not observed out of the SLZ cave in 2013.

Land use decisions, such as unsanctioned quarrying, alteration of hydrology, illegal logging, and increased tourism, can have immediate and serious impacts on karst terrains (van Beynen *et al.*, 2012). A number of limestone cave-dwelling amphibians, such as Red-spotted toothed toad (*Oreolalax rhodostigmatus*) and Wuchuan odorous frog (*Odorrana wuchuanensis*) listed as vulnerable or critically endangered species by IUCN, are particularly sensible to such human activities.

To aid the recovery of wild population, the Chinese Ministry of Agriculture organized a series of releasing activates of CGS from 2002 and about ninety-two thousand artificially bred juveniles were introduced into the wild during 2010-2013. However, most releases of CGS failed to boost the wild populations due to the limitation of factors such as water quality, food supply, and safety (Luo *et al.*, 2009). We suggest that sustainable forestry, landfills preventing groundwater contamination, and nature reserves extension to connect breeding caves instead of releasing may be the sole measure to save this flagship species in karst habitats.

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References

Cosentino B. J., Phillips C. A., Schooley R. L., Lowe W. H., Douglas M. R. 2012. Linking extinction-colonization dynamics to genetic structure in a salamander metapopulation. Proc R Soc B, 279(1733):1575–1582

Culver D. C., Deharveng L., Bedos A., Lewis J. J., Madden M., Reddell J. R., Sket B., Trontelj P., White D. 2006. The mid-latitude biodiversity ridge in terrestrial cave fauna. Ecography, 29(97333):120–128

Culver D. C., Pipan T. 2009. The Biology of Caves and Other Subterranean Habitats. Oxford: Oxford University Press

Evanno G., Regnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Mol Ecol, 14(8): 2611–2620

Fang Y., Zhang Y., Xiao H., Yang Y. 2008. Genetic diversity analysis of wild Chinese giant salamander (*Andrias davidianus*) and their artificially propagated progenies. Acta Hydrobiologica Sinica, 32(5): 783–786

Frankham R. 2005. Genetics and extinction. Biol Conserv, 126(2): 131–140

Garza J. C., Williamson E. G. 2001. Detection of reduction in population size using data from microsatellite loci. Mol Ecol, 10(2): 305–318

Goricki S., Niemiller M. L., Fenolio D. B. 2012. Salamanders. 665–676. In: White W.H., Culver D.C. (Eds), Encyclopedia of Caves, 2nd Edition. Elsevier

Guindon S., Dufayard J. F., Lefort V., Anisimova M., Hordijk W., Gascuel O. 2010. New Algorithms and methods to estimate maximum-likelihood phylogenies: accessing the performance of PhyML 3.0. Syst Biol, 59(3): 307–321

Guo S. W., Thomson E. A. 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics, 48(2): 361–372

Hoban S. M., Gaggiotti O. E., Bertorelle G. 2013. The number of markers and samples needed for detecting bottlenecks under realistic scenarios, with and without recovery: a simulation-based study. Mol Ecol, 22(13): 3444–3450

Isaac N. J. B., Redding D. W., Meredith H. M., Safi K. 2012. Phylogenetically-informed priorities for amphibian conservation. PLoS ONE, 7(8), e43912

Jamieson I. G., Allendorf F. W. 2012. How does the 50/500 rule apply to MVPs? Trends Ecol Evol, 27(10): 578–584

Jiang Z. G., Jiang J. P., Wang Y. Z., Zhang E., Zhang Y. Y., Li L. L., Xie F., Cai B., Cao L., Zheng G. M., Dong L., Zhang Z. W., Ding P., Luo Z. H., Ding C. Q., Ma Z. J., Tang S. H., Cao W. X., Li C. W., Hu H. J., Ma Y., Wu Y., Wang Y. X., Zhou K. Y., Liu S. Y., Chen Y. Y., Li J. T., Feng Z. J., Wang Y., Wang B., Li C., Song X. L., Cai L., Zang C. X., Zeng Y., Meng Z. B., Fang H. X., Ping X. G. 2016. Red list of China's vertebrates. Biodiv Sci, 24(5): 500–551

Jones O. R., Wang J. 2010. COLONY: A program for parentage and sibship inference from multilocus genotype data. Mol Ecol Resour, 10(3): 551–555

Köhler J., Vences M., D'Cruze N., Glaw F. 2010. Giant dwarfs: discovery of a radiation of large-bodied 'stump-toed frogs' from karstic cave environments of northern Madagascar. J Zool, 282(1): 21–38

Kuhner M. K. 2006. LAMARC 2.0: maximum likelihood and Bayesian estimation of population parameters. Bioinformatics, 22(6): 768–770

Liang G., Geng B., Zhao E. 2004. *Andrias davidianus*. The IUCN Red List of Threatened Species. Version 2014. 2

Librado P., Rozas J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25(11): 1451–1452

Liu J., Zhang Z., Zhang Z., Liu X., Zhang L., Zhang J. 2009. A critically endangered cave-dwelling frog—the habitat, status and conservation of *Odorrana wuchuanensis* in Guizhou, China. Bull Biol, 44(5): 14–16 (in Chinese)

Liu J. 2010. Preliminary study on the development and ecology of Red-spotted toothed toad (*Oreolalax rhodostigmatus*). Bulletin of Biology, 45(1): 50–52 (in Chinese)

Luikart G., Sherwin W. B., Steele B. M., Allendorf F. W. 1998. Usefulness of molecular markers for detecting population bottlenecks via monitoring genetic change. Mol Ecol, 7(8): 963–974

Luo Q., Liu Y., Zhang L., Chen G., Kang L. 2009. Investigation on resources of Chinese giant salamanders in Zhangjiajie City. Sichuan J Zool, 28(3): 422–436 (in Chinese)

Matsui M., Tominaga A., Liu W.Z., Tanaka-Ueno T. 2008. Reduced genetic variation in the Japanese giant salamander, *Andrias japonicus* (Amphibia: Caudata). Mol Phylogenet Evol, 49(1): 318–326

Meng Y., Yang Y., Zhang Y., Xiao H. 2008. A comparison of genetic diversity between wild and cultured populations of the Chinese giant salamander, *Andrias davidianus*, based on microsatellite analyses. Biodiv Sci, 16(6): 533–538 (in Chinese)

Murphy R.W., Fu J.Z., Upton D.E., Lema T.D., Zhao E.M. 2000. Genetic variability among endangered Chinese giant salamanders, *Andrias davidianus*. Mol Ecol, 9(10): 1539–1547

Niemiller M. L., Miller B. T. 2009. A survey of the cave-associated amphibians of the eastern United States with an emphasis on salamanders. Proceedings of the 15th International Congress of Speleology, Kerrville, Texas, 15: 249–256

Peery M. Z., Kirby R., Reid B.N., Stoelting R., Doucet-Bëer E., Robinson S., Vásquez-Carrillo C., Pauli J.N., Palsbøll P. J. 2012. Reliability of genetic bottleneck tests for detecting recent population declines. Mol Ecol, 21(14): 3403–3418

Peterman W. E., Anderson T. L., Drake D. L., Ousterhout B. H., Semlitsch R. D. 2014. Maximizing pond biodiversity across the landscape: a case study of larvae ambystomatid salamanders. Anim Conserv, 17(3): 275–285

Piry S., Luikart G., Cornuet J. M. 1999. BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. J Hered, 90(4): 502–503

Poschadel J.R., Möller D. 2004. A versatile field method for tissue sampling on small reptiles and amphibians, applied to pond turtles, newts, frogs and toads. Conserv Genet, 5(6): 865-867

Pritchard J.K., Stephens M., Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics, 155(2): 945-959

Radwan J., Biedrzycka A., Babik W. 2010. Does reduced MHC diversity decrease viability of vertebrate populations? Biol Conserv, 143(3): 537–544

Rice W. R. 1989. Analyzing tables of statistical tests. Evolution, 43(2): 223–225

Rodrigáñez J., Barragán C., Alves E., Gortázar C., Toro M. A., Silió L. 2008. Genetic diversity and allelic richness in Spanish wild and domestic population estimated from microsatellite markers. Span J Agric Res, 6 (Special Issue): 107–115

Rousset F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour 8(1): 103–106

Sabatino S. J., Routman E. J. 2009. Phylogeography and conservation genetics of the hellbender salamander (*Cyptobranchus alleganiensis*). Conserv Genet, 10(5): 1235–1246

Schwartz M. K., Luikart G., Waples R. S. 2007. Genetic monitoring as a promising tool for conservation and management. Trends Ecol Evol, 22(1): 25–33

Sket B. 1997. Distribution of Proteus (Amphibia: Urodela: Proteidae) and its possible explanation. J Biogeogr, 24(3): 263–280

Storfer A., Mech S.G., Reudink M. W., Lew K. 2014. Inbreeding and strong population subdivision in an endangered salamander. Conserv Genet, 15(1): 137–151

Su H., Yu L., Ma J. 2009. Population status and history dynamics of wild Chinese giant salamander (*Andrias davidianus*) in Yanxia Natural Reserve in Guizhou province, China. Resources and Environment in the Yangtze Basin, 18(7): 652–657. (in Chinese)

Sutton J. T., Nakagawa S., Robertson B. C., Jamieson I. G. 2011. Disentangling the roles of natural selection and genetic drift in shaping variation at MHC immunity genes. Mol Ecol, 20(21): 4408–4420

Suzuki N., Kuwabara K., Ashikaga K., Nakanishi M., Minamigata N., Morimoto H. 2000. Breeding of the Japanese giant salamander, *Andrias japonicus*, in a portable artificial nest. J Jpn Assoc Zool Gardens Aquarium, 41: 83–87 (in Japanese)

Tajima F. 1989. The effect of change in population size on DNA polymorphism. Genetics, 123(3): 597–601

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol, 30(12): 2725–2729

Tao F., Wang X., Zheng H., Fang S. 2005. Genetic structure and geographic subdivision of four populations of the Chinese giant salamander (*Andrias davidianus*). Zool Res, 26(2):162–167 (in Chinese)

Tao F., Wang X., Zheng H. 2006. Analysis of complete cytochrome b sequences and genetic relationship among Chinese giant salamanders (*Andrias davidianus*) from different areas. Acta Hydrobiol Sin, 30(5): 625–628 (in Chinese)

Tracy L. N., Wallis G. P., Efford M. G., Jamieson J. G. 2011. Preserving genetic diversity in threatened species reintroductions: How many individuals should be released? Anim Conserv, 14(4): 439–446

van Beynen P., Brinkmann R., van Beynen K. 2012. A sustainability index for karst environments. J Cave Karst Stud, 74(2): 221–234

Van Oosterhout C., Hutchinson W. F., Wills D. P. M., Shipley P. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes, 4(3): 535–538

Wang X., Zhang K., Wang Z., Ding Y., Wu W., Huang S. 2004. The decline of the Chinese giant salamander *Andrias davidianus* and implication for its conservation. Oryx, 38(2):197–202

Wang W., Jiang F., Wang B. 2000. Survey the reproductive habits of Chinese giant salamander. Res Fish, 20(6):12–13 (in Chinese)

Wang J., Zhang H.X. 2012. An ill-fated life of the larvae of Chinese giant salamander. China Nature, 168:72-73. (in Chinese)

Wang J., Zhang J., Li X., Jiang J. 2014. Isolation and characterization of 16 microsatellite loci for the giant salamander *Andrias davidianus*. Conserv Genet Resour, 6(2): 367–368

Weir B. S., Cockerham C. C. 1984. Estimating F-statistics for the analysis of population structure. Evolution, 38(6): 1358–1370

Weiser E. L., Grueber C. E., Jamieson I. G. 2013. Simulating retention of rare alleles in small populations to assess management options for species with different life histories. Conserv Biol, 27(2): 335–344

Wilson I. J., Weale M. E., Balding D. J. 2003. Inferences from DNA data: population histories, evolutionary processes and forensic match probabilities. J R Statist Soc A, 166(2): 155–201

Yang L., Meng Z., Liu X., Zhang Y., Huang J., Huang J., Lin H. 2011. AFLP analysis of five natural populations of *Andrias davidianus*. Acta Sci Nat Univ Sunyatseni, 50(2): 99–104

Zhang P., Wake D. B. 2009. High-level salamander relationships and divergence dates inferred from complete mitochondrial genomes. Mol Phylogenet Evol, 53(2): 492–508